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Further New Milbemycin Antibiotics from *Streptomyces bingchenggensis*

Fermentation, Isolation, Structural Elucidation and Biological Activities

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Abstract Three new α-class milbemycins from *Streptomyces bingchenggensis*, named milbemycins α_{28}, α_{29} and α_{30}, have been isolated and characterized. On the basis of detailed spectroscopic analysis and comparison with reported data, their structures were determined to be the 22α-hydroxy, 23β-*O*-(2-methylbutanoyl) milbemycin α_9, 22α-hydroxy, 23β-*O*-(2,4-dimethylpentanoyl) milbemycin α_9 and 22α-hydroxy, 23β-*O*-(2-methylbutanoyl) milbemycin A_4, respectively. Milbemycins α_{28} and α_{29} are the first reported milbemycins that possess both C-26 and C-23 substituents. These three new milbemycins possess potent acaricidal and nematocidal activity.

Keywords new milbemycins α_{28}, α_{29}, α_{30}, *Streptomyces bingchenggensis*, acaricidal activity, nematocidal activity

Introduction

Milbemycins and avermectins, members of the class of sixteen membered macrolides that possess potent antiparastic activity, were reviewed by Davies and Green in 1986 [1]. After the first review on milbemycins and avermetins by Davies and Green, many new families of antiparasitic macrolide antibiotics have been isolated from other microorganisms, including *Streptomyces cyaneogriseus* subsp. *noncyanogenus* [2], *Streptomyces thermoarchaensis* [3], *Streptomyces hygroscopicus* [4], *Streptomyces* sp. E225 (NCIB 12310) [5~7], *Streptomyces hygroscopicus* subsp. *aureolacrimosus* [8, 9], *Streptomyces* NCIB 11876 [10], *Streptomyces hygroscopicus* ATCC53718 [11] and a hybrid microorganism obtained by protoplast fusion of *Streptomyces avermitilis* and *S. hygroscopicus* [12]. Twelve β-type milbemycin compounds and 27 α-type milbemycin compounds from these microorganisms have been reported [13, 14].

The potent anthemintic activity exhibited by milbemycins continues to spur research to find new natural products and new semi-synthetic milbemycins with improved properties [15~22]. We have previously reported the isolation and structure elucidation of milbemycins β_{13} and β_{14} from a new *Streptomyces bingchenggensis* [23]. We investigated the fermentation broths of this microorganism in more detail and have isolated three additional new metabolites. In this paper, we describe the fermentation, isolation, structural elucidation and acaricidal activities of the three new milbemycins. Milbemycins α_{28} and α_{29} (**1** and **2**, Fig. 1) of these compounds are the first reported milbemycins that possess both C-26 and C-23 substituents. Moreover, they are highly active.

Materials and Methods

Microorganism

The producing organism, *S. bingchenggensis*, was isolated from a soil sample collected in Harbin, China. *S. bingchenggensis* has been deposited at the China General Microbiology Culture Collection Center (Accession No: CGMCC1734), and we have determined the 16S rDNA sequence (Accession No: DQ449953 in National Center for Biological Information).

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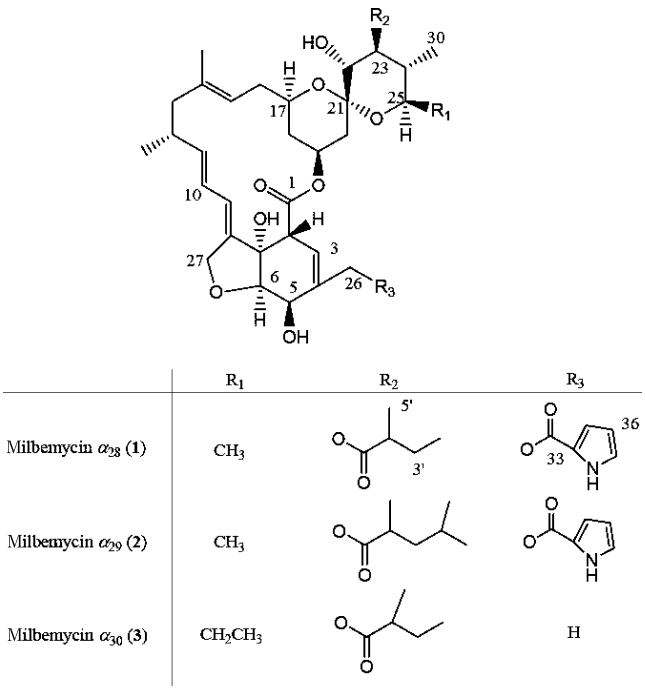

Fig. 1 The structures of milbemycin α_{28} (**1**), milbemycin α_{29} (**2**) and milbemycin α_{30} (**3**).

Fermentation

The seed for preculture was spores. The medium for sporulation contained sucrose 4.0 g, yeast extract 2.0 g, malt extract 5.0 g, skim milk 1.0 g in 1.0 liter water. The pH was adjusted to 7.0 with 1 M NaOH, 20 g of agar added, and this mixture sterilized at 121°C for 30 minutes. The spore suspension was prepared from agar plates incubated at 28°C for 7～8 days.

A spore suspension (1.0 ml) of the culture of *S. bingchenggensis* was transferred to a 250-ml Erlenmeyer flask that contained 25 ml of the seed medium containing sucrose 0.25 g, polypeptone 0.1 g, and K_2HPO_4 1.25 mg. The inoculated flasks were incubated at 28°C for 42 hours on a rotary shaker at 250 rpm. Then 8.0 ml of the culture was transferred into a 1-liter Erlenmeyer flask containing 100 ml of the producing medium consisting of sucrose 8.0%, soybean powder 1.0%, yeast extract 0.2%, meat extract 0.1%, $CaCO_3$ 0.3%, K_2HPO_4 0.03%, $MgSO_4 \cdot 7H_2O$ 0.1%, $FeSO_4 \cdot 7H_2O$ 0.005%, pH 7.2 before sterilization. Fermentation was carried out at 28°C for 8 days on a rotary shaker at 250 rpm.

Isolation and Purification

The fermentation broth (10 liters) was filtered. The resulting cake was washed with water, and the both filtrate and wash were discarded. MeOH (10 liters) was used to extract the washed cake. The MeOH extract was concentrated to approximately 2 liters under reduced pressure, and the resulting concentrate was extracted three times with an equal volume of EtOAc. The combined EtOAc phase was concentrated under reduced pressure to yield 25 g of oily substance. The residual oily substance was chromatographed on silica gel, eluting with petroleum ether - Me_2CO (95 : 5～50 : 50). Fractions not containing milbemycins A_3 and A_4 were combined to give 10 g of crude sample upon evaporation of the solvents. The crude sample was applied to a silica gel column and eluted with petroleum ether - Me_2CO (9 : 1～3 : 1) to give five fractions. Fraction 4 was further separated by RP-C_{18} silica gel column chromatography, eluting with MeOH - H_2O (70 : 30～85 : 15), to yield **1** (20 mg) and **2** (11 mg). Fraction 3 was also purified by RP-C_{18} silica gel column chromatography, eluting with MeOH - H_2O (70 : 30～95 : 5), to yield **3** (22 mg).

General

UV spectra were obtained on a Varian CARY 300 BIO spectrophotometer; IR spectra were recorded on a Nicolet Magna FT-IR 750 spectrometer (ν_{max} in cm^{-1}); ^1H- and ^{13}C-NMR spectra were measured with a Bruker DRX-400 (400 MHz for ^1H and 100 MHz for ^{13}C) spectrometer. Chemical shifts are reported in parts per million (δ), using residual $CHCl_3$ (δ_H 7.26 ppm; δ_C 77.0) as an internal standard, with coupling constants (J) in Hz. ^1H- and ^{13}C-NMR assignments were supported by ^1H-^1H COSY, HMQC and HMBC experiments. The ESI-MS and HRESI-MS spectra were taken on a Q-TOF Micro LC-MS-MS mass spectrometer. Optical rotation was measured on a Perkin-Elmer 341 Polarimeter. Commercial silica gel (Qing Dao Hai Yang Chemical Group Co., 100～200 and 200～300 mesh) and reverse phase C_{18} silica gel were used for column chromatography. Spots were detected on TLC under UV or by heating after spraying with sulfuric acid - ethanol, 5 : 95 (v/v).

Antiparasitic Activity

Acaricidal Activity against Adult Mites

MeOH solutions containing 0.1% of individual compounds were diluted 10-fold with water containing 0.01% of detergent to prepare 100 μg/ml solutions. Then appropriate further dilutions were prepared. Two-spotted spider mites, sensitive to organophosphorus insecticides, were inoculated on the primary leaves of cowpea plants. One day after inoculation, leaves of cowpea plants were soaked in the sample solutions for 1～2 seconds and the leaves were kept at 25°C. After 3 days, survival of the adult insects was determined with a binocular microscope and the mortality (%) was calculated.

Acaricidal Activity against Mite Eggs

Sample solutions containing 100, 50, 30, and 10 μg/ml of individual compounds were prepared. Female adult two-spotted spider mites were allowed to lay eggs on the primary leaves of cowpea plants. The adult mites were removed to obtain test leaves each bearing about 40 eggs. In a similar manner to the preceding example, the test leaves were soaked in the sample solutions for 1~2 seconds. After 10 days at 25°C, the number of unhatched eggs was counted, and the unhatched egg rates (%) were calculated.

Nematocidal Activity

MeOH solutions containing 0.1% of individual compounds were diluted 10-fold with water to prepare solutions containing 100 μg/ml. Then appropriate amounts of the solutions were added to 1-ml portions of an aqueous suspension containing living nematodes, *Caenorhabditis elegans*. The mixtures were left at 25°C for 15 hours after shaking. The number of nematodes that were immobilized and the total number of the nematodes tested were counted under a stereoscopic microscope. Immobilized rates (%) against the total number of tested nematodes were calculated.

Results and Discussion

Physico-chemical Properties of 1 , 2 and 3

1 (Fig. 1) $C_{41}H_{55}NO_{12}$, white amorphous powder; $[\alpha]_D^{20}$ +108° (*c* 0.65, $CHCl_3$); UV (MeOH) λ_{max} nm (log ε): 244 (4.54), 238 (4.50); IR (KBr), ν_{max} cm^{-1}: 3455 (OH), 1712; ^{1}H-NMR ($CDCl_3$, 400 MHz) and ^{13}C-NMR ($CDCl_3$, 100 MHz) data see Table 1; ESI-MS *m/z* 771 $[M+NH_4]^+$; HRESI-MS *m/z* 771.4067, calcd for $C_{41}H_{59}N_2O_{12}$ 771.4068.

2 (Fig. 1) $C_{43}H_{59}NO_{12}$, white amorphous powder; $[\alpha]_D^{20}$ +108° (*c* 0.65, $CHCl_3$); UV (MeOH) λ_{max} nm (log ε): 244 (4.54), 238 (4.50); IR (KBr), ν_{max} cm^{-1}: 3465 (OH), 1710; ^{1}H-NMR ($CDCl_3$, 400 MHz) and ^{13}C-NMR ($CDCl_3$, 100 MHz) data see Table 1; ESI-MS *m/z* 799 $[M+NH_4]^+$; HRESI-MS *m/z* 799.4380, calcd for $C_{43}H_{63}N_2O_{12}$ 799.4381.

3 (Fig. 1) $C_{37}H_{54}O_{10}$, white amorphous powder; $[\alpha]_D^{20}$ +108° (*c* 0.65, $CHCl_3$); UV (MeOH) λ_{max} nm (log ε): 246 (4.54) , 238 (4.49); IR (KBr), ν_{max} cm^{-1}: 3425 (OH), 1720, ^{1}H-NMR ($CDCl_3$, 400 MHz) and ^{13}C-NMR ($CDCl_3$, 100 MHz) data see Table 1; ESI-MS *m/z* 657 $[M-H]^-$; HRESI-MS *m/z* 657. 3638, calcd for $C_{37}H_{53}O_{10}$ 657.3639.

Structure Elucidation

The structures of the three new milbemycins were determined by the analysis and the comparison of ^{1}H-NMR, ^{13}C-NMR, MS, and IR data with those of milbemycin α_9 [18] and 22-hydroxy-23-*O*-(2-methylbutanoyl) milbemycin A_3 [10]. The ^{1}H-and ^{13}C-NMR data of new milbemycins are summarized in Table 1. The molecular formulas were established from the HRESI-MS spectra. In the ^{1}H- and ^{13}C-NMR spectra of the three new milbemycins, signals corresponding to the 16-membered macrolide structures were found. The structural differences between **1**, **2** and milbemycin α_9 were found in the substitutions at positions 22 and 23. The ethyl substitution at position 25 was the only difference between **3** and 22-hydroxy-23-*O*-(2-methylbutanoyl) milbemycin A_3.

By detailed comparison the ^{1}H- and ^{13}C-NMR data of **1** and **2** with those of milbemycin α_9, it was shown that **1** and **2** were the derivatives of milbemycin α_9, in which a hydroxyl and an alkanoyloxyl were substituted at positions 22 and 23, respectively. In **1**, the correlation of H-22 (δ_H 3.22) and H-23 (δ_H 4.93) in ^{1}H-^{1}H COSY experiment and the HMBC correlation signals observed between H-30 (δ_H 0.84) and C-23 (δ_C 75.4), H-23 and the ester carbonyl at 177.5, indicated the hydroxyl was at position 22 and the alkanoyloxyl was at position 23. By analysis of the ^{1}H-^{1}H COSY and the HMBC correlation signals, combined with the MS data, it was showen that the substitution at position 23 was 2-methylbutanoyloxyl. So the structure of **1** was established. In similar fashion, **2** was elucidated as 22-hydroxy-23-*O*-(2,4-dimethylpentanoyl) milbemycin α_9.

Comparison of the ^{1}H-NMR data of **3** with that of 22-hydroxy-23-*O*-(2-methylbutanoyl) milbemycin A_3 [10], indicated the difference between them was only the substitution at position 25, where a methyl was replaced by an ethyl. The 14 mass unit molecular weight enhancement of **3** compared with that of 22-hydroxy-23-*O*-(2-methylbutanoyl) milbemycin A_3 further confirmed the structure of **3**. The structure of **3** was thus established as 22-hydroxy-23-*O*-(2-methylbutanoyl) milbemycin A_4.

The relative stereochemistry of the C-22 and C-23 substituents in the three milbemycins was assigned by NOESY experiments. Unambiguous assignment of the relative stereochemistry shown in Fig. 1 was obtained from the observation of NEOSY correlation signals between protons H_{22} and H_{24} as well as H_{23} and H_{25}. The C-2′ stereochemistry in the C_{23} (R_2) substituents of the compounds remained unsolved and have not been reported in the related milbemycins [10, 25~28].

Biological Activity

Three new milbemycins **1**~**3** possess potent acaricidal and nematocidal activity (Tables 2 and 3). Takahashi [24] reported C-26 substituted milbemycins possessing high

Table 1 ^{1}H- and ^{13}C-NMR data of milbemycins α_{28} (**1**), α_{29} (**2**), and α_{30} (**3**) (coupling constants in parenthesis)

Position	Proton			Carbon		
	1	**2**	**3**	**1**	**2**	**3**
1				172.9 s*	172.9 s*	173.5 s*
2	3.36 br s	3.37 br s	3.28 br s	45.6 d	45.7 d	45.7 d
3	5.80 br s	5.82 br s	5.42 br s	121.7 d	121.7 d	118.1 d
4				136.5 s	136.6 s	137.9 s
5	4.55 d (6.0)	4.55 d (6.0)	4.32 d (6.6)	64.7 d	64.8 d	67.7 d
6	4.01 d (6.0)	4.01 d (6.0)	3.97 d (6.6)	79.2 d	79.3 d	79.2 d
7				80.4 s	80.4 s	80.2 s
8				139.1 s	139.2 s	139.6 s
9	5.80 d (11.3)	5.82 d (11.2)	5.79 d (11.3)	120.6 d	120.6 d	120.3 d
10	5.76 dd (14.0, 11.3)	5.77 dd (14.0, 11.2)	5.77 dd (14.5, 11.3)	123.4 d	123.3 d	123.4 d
11	5.38 dd (14.0, 10.0)	5.38 dd (14.0, 10.0)	5.38 dd (14.5, 10.0)	143.1 d	143.0 d	142.8 d
12	2.45 m	2.44 m	2.43 m	36.0 d	36.0 d	35.9 d
13	2.24 m	2.23 m	2.20 m	48.5 t	48.5 t	48.5 t
	1.93 m	1.89 m	1.88 m			
14				137.5 s	137.4 s	137.5 s
15	4.98 m	4.97 m	4.94 m	120.3 d	120.4 d	120.3 d
16	2.24 m	2.25 m	2.24 m	34.7 t	34.8 t	34.5 t
17	3.65 m	3.63 m	3.61 m	68.5 d	68.5 d	68.5 d
18	1.86 m	1.85 m	1.83 m	36.2 t	36.1 t	36.2 t
	0.91 m	0.93 m	0.93 m			
19	5.33 m	5.33 m	5.34 m	68.5 d	68.4 d	68.0 d
20	1.93 m	1.93 m	1.92 m	36.1 t	36.0 t	36.3 t
21				100.0 s	100.0 s	99.8 s
22	3.22 br t (8.8)	3.24 br t (8.8)	3.22 m	75.4 d	75.4 d	75.4 d
23	4.93 m	4.93 m	4.94 m	75.4 d	75.5 d	75.4 d
24	1.55 m	1.57 m	1.64 m	42.2 d	42.3 d	39.9 d
25	3.45 m	3.44 m	3.22 m	69.3 d	69.4 d	73.7 d
26	4.98 d (13.4)	4.99 d (13.4)	1.88 s	64.2 t	64.1 t	19.9 q
	4.88 d (13.4)	4.87 d (13.4)				
27	4.71 br s	4.71 br s	4.70 d (14.5)	68.4 t	68.4 t	68.4 t
			4.66 d (14.5)			
28	1.02 d (6.7)	1.03 d (6.7)	1.01 d (6.8)	22.3 q	22.2 q	22.3 q
29	1.55 br s	1.56 br s	1.55 br s	15.6 q	15.5 q	15.6 q
30	0.84 d (6.5)	0.85 d (6.5)	0.84 d (6.6)	13.0 q	13.0 q	12.7 q
31	1.22 d (6.2)	1.23 d (6.2)	1.75 m	18.8 q	18.8 q	25.1 t
			1.45 m			
32			1.03 t (6.8)			9.9 q
33				160.9 s	160.8 s	
34				122.2 s	122.3 s	
35	6.97 d (5.8)	6.97 d (5.8)		116.0 d	115.9 d	
36	6.27 dd (5.8, 2.9)	6.27 dd (5.8, 2.9)		110.5 d	110.5 d	
37	6.98 t (2.9)	6.98 t (2.9)		123.4 d	123.4 d	
1′				177.5 s	177.8 s	177.5 s
2′	2.45 m	2.59 m	2.43 m	41.5 d	38.0 d	41.6 d
3′	1.71 m	1.65 m	1.75 m	26.8 t	42.9 t	26.8 t
	1.52 m	1.23 m	1.50 m			
4′	0.95 t (7.4)	1.65 m	0.95 t (7.5)	11.6 q	25.9 d	11.7 q
5′	1.19 d (7.0)	0.90 d (7.0)	1.19 d (6.9)	16.8 q	22.5 q	16.8 q
6′		1.18 d (7.0)			17.7 q	
7′		0.88 d (7.0)			22.6 q	

* By DEPT sequence.

Table 2 Acaricidal activity of milbemycins against adult mites and mite eggs

Concentration (μg/ml)	Adult mites mortality (%)				Mite eggs unhatched (%)			
	α_{28}	α_{29}	α_{30}	A_3/A_4*	α_{28}	α_{29}	α_{30}	A_3/A_4*
100	100	100	100	100	34.6	44.6	57.9	43.5
50	100	100	87.2	100	33.5	23.5	37.6	17.3
30	100	100	89.4	89.3	13.7	15.7	32.4	4.1
10	100	95.6	81.7	73.5	0	0	10.4	
2	89.3	90.3	43.5	23.5				

* Milbemycins A_3 and A_4 mixtures, 30 : 70 (in volume).

Table 3 Nematocidal activity of milbemycins against *Caenorhabditis elegans*

Concentration (μg/ml)	Immobility (%)			
	α_{28}	α_{29}	α_{30}	A_3/A_4*
100	100	100	100	100
50	100	100	100	100
30	100	100	90	93
10	89	96	67	73
2	77	75	35	26

* Milbemycins A_3 and A_4 mixtures, 30 : 70 (in volume).

activity. Poole [10] described C-23 substituted milbemycins also with high activity. However, the high activity with both C-26 and C-23 substituted milbemycins has been first demostrated in this paper.

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